Growth Inhibition of Human Colon Cancer Cell Line HCT116 by Bis[2-(acylamino)phenyl] Disulfide and Its Action Mechanism

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Our laboratory has been investigating the use of compounds which disrupt β-catenin/T cell factor (TCF) binding to treat human colon cancer. There are several cysteine residues on the surface of β-catenin where it binds to TCF. Some bis[2-(acylamino)phenyl] disulfides might have the ability to form a disulfide bond with the cysteine residues of β-catenin, leading to inhibition of the growth of human colon cells. Bis[2-(acylamino)phenyl] disulfides were screened to inhibit the growth of cancer cells. Among them, bis[2-(2,2-dimethylpropanoylamino)phenyl] disulfide (1) had promising inhibitory effects (HCT116, IC50: 9.7 μM; DLD-1, IC50: 6.9 μM) on cell proliferation, and did not show any cytotoxicity among normal human fibroblast CCD-1059SK cells even at 200 μM. This derivative reduced the β-catenin/TCF4 association in the HCT116 cells to ca. 50% at 150 μM. Furthermore, it activated markedly the phosphorylation of c-Jun N-terminal kinase (JNK) connected to stress-activated apoptosis at a lower concentration (30 μM). In view of cell cycle analyses, Hoechst staining, and terminal deoxynucleotidyl transferase-mediated dUTP-biotin Nick end-labeling (TUNEL) assays along with the above results, it is likely that 1 inhibited the growth of HCT116 cells through pathways including the JNK-mediated apoptosis.

Key words HCT116; bis[2-(acylamino)phenyl] disulfide; c-Jun N-terminal kinase; apoptosis; β-catenin/T cell factor association
Japan). Propidium iodide was from Sigma (MI, U.S.A.). The anti-TCF4 antibody was from Upstate Biotechnology (NY, U.S.A.), and the other antibodies were from Sigma (MI, U.S.A.). The HRP-linked secondary antibody, ECL-Plus reagents, and Protein G were from GE Healthcare UK Ltd. (Buckingham, U.K.). The Cyclotest Plus DNA reagent Kit was purchased from Becton Dickinson and Company (NJ, U.S.A.). The TUNEL assay reagent, DeadEnd™ TUNEL Kit (DeadEnd™ Fluorometric TUNEL System), was obtained from Promega (WI, U.S.A.). Results of flow cytometry were recorded with FACSscan (Becton Dickinson and Company). Apoptosis was observed with a Nikon ECLIPSE TE-2000U.

**Cell Culture**

HCT116 cells were cultured in McCoy’s 5A medium supplemented with 10% fetal bovine serum (FBS), 50 μg/ml penicillin G, and 50 μg/ml streptomycin sulfate (Invitrogen, U.S.A.) in a 5% CO2 and 95% atmosphere at 37°C. CDD-1059SK cells were cultured in Earle’s medium supplemented with 10% FBS, 1% l-glutamine–200 mM, and 1% MEM Non-Essential Amino Acids Solution–10 mM in a 5% CO2 and 95% atmosphere at 37°C.

**Cell Viability Assay**

HCT116 and DLD-1 cell lines were cultured in RPMI 1640 medium containing 10% FBS (Moregate Bio Tech, Australia). The proliferation assay of 18 against HCT116 and DLD-1 cells was assessed by the conventional methylene blue staining method, whereas that of NCX-4040 (2)19 (Fig. 1) was by the conventional WST-1 assay.19

**Hoechst Staining**

HCT116 cells were plated onto 60 mm diameter dishes (1.0×106 cells/dish) and allowed to grow for 24 h. After incubation with H2O2 (150 μM) or disulfide 1 (30, 60, 120 μM) for 48 h, cells were fixed in 2% glutaraldehyde for 4 h and washed twice with PBS before being stained with 1 μg/ml Hoechst 33342 for 30 min under ice-cooling in the dark. Cells were then washed twice with PBS. Apoptosis, with condensed and fragmented nuclei, was observed with a fluorescence microscope.

**TUNEL Assay by Flow Cytometry**

HCT116 cells were plated onto 60 mm diameter dishes (1.0×106 cells/dish). After incubating for 24 h, the cells were incubated with disulfide 1 (30, 60, 120 μM) for 48 h. Adhered and floating cells were subjected to a TUNEL assay using the DeadEnd™ Fluorometric TUNEL System according to the procedure recommended in the Promega DeadEnd™ TUNEL Kit in the following way: The cells in which DNA fragments obtained by apoptosis were bound to fluorescein-12-dUTP were stained with propidium iodide to be analyzed by flow cytometry. For each sample, 20000 events were recorded.

**Western Blot Analysis**

HCT116 cells were plated onto 60 mm diameter dishes (1.0×106 cells/dish). After incubating for 24 h, the cells were washed twice with the serum-free medium (1 ml). After addition of the serum-free medium (5 ml), the culture was incubated for another 24 h and incubated with disulfide 1 (30 μM) for 0, 1/12, 1/4, 1/2, 1, 2, 4, 6, 8, 12, and 24 h. Adhered and collected cells, washed twice with PBS, and resuspended in 400 μl of the modified Covance Laboratoes lysis buffer21 (pH 8.0 and 1% Nonidet P-40 were adopted in place of pH 7.4 and 1% Triton-X 100, respectively). The lysates were centrifuged at 14000×g for 15 min at 4°C. The protein concentration was determined with a Bio-Rad protein assay kit. An equal amount of protein was then resolved by SDS-PAGE and transferred to PVDF membrane. Primary monoclonal antibodies were against the following proteins at a 1:2500 dilution: actin, β-catenin, cyclin D1, ERK, p-ERK, JNK, p-JNK, p21/WAF1, p38, p-p38, and TCF4. Secondary antibodies conjugated to horseradish peroxidase (1:2500) were from Sigma. Immunoreactive protein was detected by using ECL chemiluminescence.

**Immunoprecipitation**

HCT116 cells were plated onto 60 mm diameter dishes (1.0×106 cells/dish). After incubating for 24 h, the cells were washed twice with the serum-free medium (1 ml). After addition of the serum-free medium (5 ml), the culture was incubated for another 24 h and incubated with NCX-4040 (2) (10, 20 μM) or disulfide 1 (150, 300 μM) for 18 h. Adhered and floating cells were collected, washed twice with PBS and resuspended in 400 μl of lysis buffer (1 M Tris–HCl (pH 8.0) 1 ml, 5 mM NaCl, 1% Nonidet P-40, 1 mg/ml aprotinin, 1 mg/ml leupeptin, 1 mg/ml pepstatin, 200 mM Na3VO4, 200 mM NaF, 200 mM NaH2PO4, 15000×g for 15 min at 4°C. The protein concentration was determined with a Bio-Rad protein assay kit. Anti-TCF4 monoclonal antibody (2.5 μl) and 600 μl of Protein G-Sepharose (2 mg/ml) were added to 200 μg (220 μl) of the protein solution in the above-mentioned buffer and incubated at 4°C for 1 h. The beads were washed three times with this lysis buffer, and the proteins were dissolved in the loading buffer for SDS/PAGE. The membrane was incubated successively with primary antibody and horseradish peroxidase-conjugated secondary antibody in the conventional way. Final detection was performed with ECL Plus reagents.

**Analysis of Cell Cycle Regulation by Flow Cytometry**

HCT116 cells were plated onto 60 mm diameter dishes (1.0×106 cells/dish) and allowed to grow for 24 h. The cells were incubated with disulfide 1 (30, 60, 120 μM) for 48 h. Adhered and floating cells were collected and then treated with a BD CycleTest Plus DNA reagent Kit (#340242) according to the procedure recommended by Becton Dickinson. DNA content was measured with a FACSscan.

**RESULTS**

**Disulfide 1 Inhibited the Growth of the HCT116 Cells**

Disulfide Bis[2-(acylamino)phenyl] disulfides were screened for anti-proliferative activity against cancer cells. Among them, disulfide 1 showed promising inhibitory effects against HCT116 (IC50: 9.7 μM) and DLD-1 cells (IC50: 6.9 μM). Notably, this compound did not show any cytotoxicity to CDD-1059SK cells even at a concentration of 200 μM. On the other hand, NCX-4040 (2), a NO-ASA derivative inhibiting β-catenin/TCF4 binding, had an IC50 of 68.0 μM against the growth of HCT116 cells.

**Disulfide 1 Induced Apoptosis**

In order to identify the apoptosis induced by disulfide 1, HCT116 cells were exposed to 1 at concentrations of 30, 60, and 120 μM for 48 h, and stained with Hoechst 33342. Apoptosis with condensed and fragmented nuclei began to be observed at 60 μM and was marked at 120 μM (Fig. 2A). This finding was also supported by the TUNEL assay using a DeadEnd™ Fluorometric TUNEL System which detected apoptotic cells at higher FL1-H levels (DNA strands labeled with the fluorescein-12-dUTP) than the scales for control (no sample) at 60 and 120 μM of 1 (Fig. 2B). This finding also supported the above-
Disulfide 1 Activated Phosphorylation of JNK

The activation of major MAPK signaling molecules, ERK, p38, and JNK, by disulfide 1 was assessed in HCT116 cells by Western blotting. As shown in Fig. 3, at 30 μM, the level of phosphorylated JNK was markedly increased, whereas those of ERK and p38 were not. The phosphorylation level of JNK protein peaked at 1—2 h.

Disulfide 1 Disrupted the β-Catenin/TCF4 Association at a High Concentration

HCT116 cells were treated with either 1 or positive control, NCX-4040 (2), as described earlier. TCF4 and β-catenin were coimmunoprecipitated from the lysates by using an anti-TCF4 antibody. Disulfide 1 reduced the association of β-catenin/TCF4 to ca. 50% at a high concentration (150 μM) (Fig. 4). In contrast, 2 disrupted significantly the association between β-catenin and TCF4; it reduced this association to ca. 30 and 10% at 10 and 20 μM with respect to the untreated control, respectively.

Disulfide 1 Did Not Cause Appreciable Modification of Cell Cycle Phases

HCT116 cells were investigated for cell

Fig. 2. Induction of Apoptosis by Disulfide 1 in HCT116 Cells

(A) Nuclear Condensation and Fragmentation: HCT116 cells (1.0 × 10⁶) were incubated for 24 h and treated with 1 for 48 h. They were processed for Hoechst 33342 staining. Nuclear staining was observed under a fluorescence microscope. Data shown are those for the control (no sample) and for the treatment with 1 (60, 120 μM) and H₂O₂ (150 μM). Shown are results representative of five independent experiments. (B) TUNEL Assay by Flow Cytometry: Cells (1.0 × 10⁶), after incubation for 24 h, were treated with 1 for 48 h. Cells were then treated with fluorescein-12-dUTP and rTdT Enzyme as well as with propidium iodide. Changes in the population of viable cells and apoptotic cells among the HCT116 cells were examined by flow cytometry. FL2-H represents DNA content, whereas FL1-H corresponds to fluorescein-12-dUTP-labeled DNA fragments. Data shown are those for the control (no sample) and for the treatment with 1 (60, 120 μM). The experiments were repeated two times with the same result.

Fig. 3. Effect of Disulfide 1 on the Activation of MAP Kinases

HCT116 cells (1.0 × 10⁶), after incubating for 24 h, were treated with 1 as indicated. The cell extracts were examined by Western blotting using respective antibodies. An antibody to actin served as a loading control. Shown are time courses of MAPK activities induced by 1 (30 μM), which are results representative of three independent experiments.
begin to be observed at 60 μm and was marked at 120 μm in the Hoechst staining test (Fig. 2A) as well as the TUNEL assay (Fig. 2B). In the immunoprecipitation assay, disulfide I reduced the association of β-catenin/TCF4 to ca. 50% with respect to the untreated control at a high concentration (150 μM). Taking into consideration the ability of 2 to disrupt significantly the β-catenin/TCF4 association in HCT116 cells to ca. 30 and 10% at lower concentrations (10, 20 μM), respectively, and to suppress the cell growth at an IC₅₀ of 68.0 μM, the inhibitory efficacy of 2 against the β-catenin/TCF4 association seems not necessarily connected to inhibition of the proliferation of HCT116 cells, as suggested in the reports (6,7) on the correlation between the growth inhibition of SW480 cells and inhibition of β-catenin/TCF signaling by 2. Thus, disulfide I should suppress the growth of HCT116 cells via other pathways. In the Western blot assay, the β-catenin-dependent expression of cyclin D1 connected to G₁/G₂ arrest was slightly down-regulated time-dependently, whereas that of p21/WAF1 linked to G₁/G₂ transition and/or G₂/M cell cycle arrest was up-regulated at 2—4 h in response to I at 30 μM. However, only a small change in the proportion of cells in G₁/G₂, S, and G₂/M was observed even at 120 μM of I using the flow cytometry. Furthermore, I activated markedly the phosphorylation of JNK connected to stress-activated apoptosis at 30 μM. The results obtained so far led us to assume that disulfide I inhibited the growth of HCT116 cells through pathways including the JNK-mediated apoptosis. The cell cycle regulation invoked by the down-regulation of cyclin D1 protein and up-regulation of p21/WAF1 could also serve to inhibit the cell growth though to little if any extent.

**DISCUSSION**

Bis[2-(2,2-dimethylpropanoylamino)phenyl] disulfide (I) inhibited the growth of HCT116 and DLD-1 cells with IC₅₀ values of 9.7 and 6.9 μM, but did not show any cytotoxicity to normal human fibroblasts, CCD-1059SK cells, even at 200 μM. Apoptosis with condensed and fragmented nuclei began to be observed at 60 μM and was marked at 120 μM in the Hoechst staining test (Fig. 2A) as well as the TUNEL assay (Fig. 2B). In the immunoprecipitation assay, disulfide I reduced the association of β-catenin/TCF4 to ca. 50% with respect to the untreated control at a high concentration (150 μM). Taking into consideration the ability of 2 to disrupt significantly the β-catenin/TCF4 association in HCT116 cells to ca. 30 and 10% at lower concentrations (10, 20 μM), respectively, and to suppress the cell growth at an IC₅₀ of 68.0 μM, the inhibitory efficacy of 2 against the β-catenin/TCF4 association seems not necessarily connected to inhibition of the proliferation of HCT116 cells, as suggested in the reports (6,7) on the correlation between the growth inhibition of SW480 cells and inhibition of β-catenin/TCF signaling by 2. Thus, disulfide I should suppress the growth of HCT116 cells via other pathways. In the Western blot assay, the β-catenin-dependent expression of cyclin D1 connected to G₁/G₂ arrest was slightly down-regulated time-dependently, whereas that of p21/WAF1 linked to G₁/G₂ transition and/or G₂/M cell cycle arrest was up-regulated at 2—4 h in response to I at 30 μM. However, only a small change in the proportion of cells in G₁/G₂, S, and G₂/M was observed even at 120 μM of I using the flow cytometry. Furthermore, I activated markedly the phosphorylation of JNK connected to stress-activated apoptosis at 30 μM. The results obtained so far led us to assume that disulfide I inhibited the growth of HCT116 cells through pathways including the JNK-mediated apoptosis. The cell cycle regulation invoked by the down-regulation of cyclin D1 protein and up-regulation of p21/WAF1 could also serve to inhibit the cell growth though to little if any extent.

**REFERENCES AND NOTES**

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18) These compounds were synthesized in the laboratory of Kansai University.


